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RESEARCH ARTICLE

Improved Detection of Germline Mutations in the von Hippel-Lindau Disease Tumor Suppressor Gene

Catherine Stolle,[1] Gladys Glenn,[2] Berton Zbar,[3]* Jeffrey S. Humphrey,[4] Peter Choyke,[5] McClellan Walther,[6] Svetlanna Pack,[7] Kathy Hurley,[6] Carolyn Andrey,[6] Richard Klausner,[8] and W. Marston Linehan[6]

[1]*Genetic Diagnostic Laboratory, University of Pennsylvania, Philadelphia, PA*
[2]*Genetic Epidemiology Branch, National Cancer Institute, Bethesda, Maryland*
[3]*Laboratory of Immunobiology, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland*
[4]*Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development*
[5]*Diagnostic Radiology, National Institutes of Health, Bethesda, Maryland*
[6]*Urologic Oncology Branch, National Cancer Institute, Bethesda, Maryland*
[7]*The Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland*
[8]*The Office of the Director, National Cancer Institute, Bethesda, Maryland*

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von Hippel-Lindau disease (VHL) is an inherited neoplastic disorder characterized by the development of tumors in the eyes, brain, spinal cord, inner ear, adrenal gland, pancreas, kidney, and epididymis. The VHL tumor suppressor gene was identified in 1993. Initial studies reported the detection of germline mutations in the VHL gene in 39–75% of VHL families. We used tests that detect different types of mutations to improve the frequency of detection of germline mutations in VHL families. The methods included quantitative Southern blotting to detect deletions of the entire VHL gene, Southern blotting to detect gene rearrangements, fluorescence in situ hybridization (FISH) to confirm deletions, and complete sequencing of the gene. Here we report that we have detected germline mutations in the VHL gene in 100% (93/93) of VHL families tested. In addition, we describe 13 novel intragenic VHL germline mutations. With the methodology described in this article, it is now possible to identify germline mutations in virtually all families with VHL. Hum Mutat 12:417–423, 1998.

KEY WORDS: **von Hippel-Lindau disease; germline mutation; pheochromocytoma; renal cell carcinoma; Southern blotting**

INTRODUCTION

von Hippel-Lindau disease (VHL) (MM #19330) is an inherited neoplastic disorder characterized by a predisposition to develop retinal angiomas, central nervous system hemangioblastomas, clear cell renal carcinomas, pheochromocytomas, pancreatic cysts and tumors, and endolymphatic sac tumors (Maher and Kaelin, 1997). The VHL gene was isolated in 1993 (Latif et al., 1993). Since that time investigators have described germline mutations in VHL families from Europe, the United States, and Asia (Whaley et al., 1994; Clinical Research Group for Japan, 1995; Chen et al., 1995; Crossey et al., 1995; Maher et al., 1996; Zbar et al., 1996; Glavac et al., 1996). A VHL gene mutation database has been established (Beroud et al., 1998).

The VHL gene regulates a number of target genes including vascular endothelial growth factor (Iliopoulos et al., 1996; Gnarra et al., 1996; Siemeister et al., 1996) transforming growth factor α (Knebelman et al., 1998), and carbonic anhydrases 9 and 12 (Ivanov et al., in press). The VHL protein may control mRNA stability through the selective degradation of RNA-bound proteins. The VHL protein participates in the regulation of cellular response to ischemia (Iliopoulos et al., 1996; Gnarra et al., 1996; Siemeister et al., 1996),

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*Correspondence to: Berton Zbar, Laboratory of Immunobiology, Building 560, Room 12-89, National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD 21702; FAX: 301-846-6145; E-mail: zbar@ncifcrf.gov

low ambient serum concentrations (Pause et al., 1998), and glucose deprivation (Gorosphe et al., submitted).

Different laboratories have reported the detection of germline mutations in the VHL gene in 39–80% of VHL families (Whaley et al., 1994; Clinical Research Group for Japan, 1995; Chen et al., 1995; Crossey et al., 1994; Maher et al., 1996; Zbar et al., 1996; Glavac et al., 1996). In our research laboratory at the National Cancer Institute-Frederick Cancer Research Facility (NCI-FCRDC, Frederick, MD), we detected germline VHL mutations in 85/114 (75%) families (Chen et al., 1995). Investigators have suggested that the VHL families in which no mutations were detected might have mutations in the VHL promoter or 3′ UTR.

Here we describe our methods for detection of germline mutations in VHL families. We have detected germline VHL gene mutations in 93/93 (100%) of VHL families. The improved ability to detect VHL gene germline mutations primarily reflected the use of quantitative Southern blotting to detect deletions of the entire VHL gene. In addition, we describe 13 novel intragenic VHL germline mutations.

MATERIALS AND METHODS

Patients

We have previously described our criteria for establishing the diagnosis of VHL, and the diagnostic tests used in patient evaluation (Glenn et al., 1990). All patients were examined at the Clinical Center of the National Institutes of Health (Bethesda, MD).

Southern Blot Analysis

Genomic DNA was extracted from whole blood using a commercially available kit (Puregene, Gentra Systems, Inc., Research Triangle Park, NC). To detect complete or partial gene deletions, five micrograms of DNA were digested overnight at 37 C with either Eco RI alone, or a combination of Eco RI and Ase I, and hybridized with the gp 7 cDNA probe. To detect a polymorphism in the VHL gene, DNA was digested with Pst I (Richards et al., 1993). Digestion products were separated by electrophoresis in a 1% agarose gel (20 cm × 20 cm) at 50 volts for 21 hr in 1X TAE running buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.3). The DNA was transferred to a nitrocellulose membrane (NitroPure, Microsystems, Inc., Westborough, MA) by capillary blotting (Southern, 1975). DNA was crosslinked to the membrane using a UV crosslinker (Stratagene, La Jolla, CA) and hybridized to random primer labeled probes specific for the VHL gene (Latif et al. 1993) and the human β globin gene (Stolle et al., 1987). Final washing conditions were 65 C in 0.2X SSC, with 0.1% sodium dodecyl sulfate (SDS). Filters were exposed to X-ray film (Kodak X-AR, Rochester, NY) in a film cassette with two intensifying screens at 70 C for 1–3 days.

In normal individuals a single 22 kb Eco RI or 9.7 kb Eco RI/Ase fragment was detected by hybridization with the gp 7 cDNA probe. In individuals with partial deletions of the VHL gene (rearrangements), a slower or faster migrating fragment was detected in addition to the normal sized fragment.

VHL Gene Deletions

Germline deletions of the entire VHL gene were detected by quantitative Southern blotting. A germline deletion of the entire VHL gene was suspected when there was a reduction of signal intensity of the fragment hybridized to the gp 7 cDNA when equal amounts of DNA were loaded per lane as determined by the signal intensity of fragments hybridized to a probe for the human β globin gene. Controls for Southern blotting included DNA from patients with complete (Yao et al., 1993) and partial deletions of the VHL gene as well as DNA from individuals without VHL.

Patients suspected of having a germline deletion of the VHL gene were tested for heterozygosity at VHL gene nucleotide 19, or for heterozygosity for a Pst I polymorphism (Richards et al. 1993). Detection of heterozygosity at either VHL nucleotide 19 or Pst I excluded a VHL germline deletion. Patients suspected of having a germline VHL deletion were further evaluated by fluorescence in situ hybridization (FISH) on established B-cell lines (Pack et al., submitted).

DNA Sequence Analysis

Genomic DNA (300 ng) was amplified by the polymerase chain reaction in a standard PCR buffer containing 200 uM dNTPs, 1.5 mM MgCl2, 0.25 units of Taq polymerase, and 0.5 μM primers using 30 cycles as follows: 95 C for 30 sec, 55 C (exons 2) or 65 C (exons 1 and 3) for 30 sec, and 72 C for 30 sec. The addition of 10% DMSO to the reaction improved amplification of exon 1. Polymerase chain reaction (PCR) products were sequenced using a cycle sequencing kit with dye-labeled terminators (PE Advanced Biosystems, Inc., Foster City, CA). Sequences were analyzed on an ABI 377 automated sequencer. Primers used for both PCR and sequencing were:

IF: CGAAGAGTACGGCCCTGAAGAAGAC;
1R: CAGTACCCTGGATGTGTCCTGCCTC;
2F: AGACGAGGTTTCACCACGTTAGC;
2R: GTCCTCTATCCTGTACTTACCAC;
K59: CACACTGCCACCATACATGCACTC;
6b: TACCATCAAAAGCTGAGATGAAACAGTGTA.

Primers used to amplify the 5′ end of exon 1 containing a polymorphism at nucleotide 19 (Chen et al., 1995) were K54: GAA ATACAG TAA CGA GTT GGC CTA GC and RD101: CCC AGC TGG GTC GGG CCT AAG CGC CGG GC. Amplification was performed with 35 cycles of 94 C for 60 sec, 60 C for 60 sec, and 72 C for 60 sec. Products were sequenced as described above.

The portion of the gene sequenced was exon 1 (nt 300–553 plus 30 bp 3′ of exon 1), exon 2 (nt 554–676 plus 30 bp 5′ and 3′ of exon 2), and exon 3 (nt 677 to the stop codon at nt 855 plus 30 bp 5′ of exon 3). Nucleotides are numbered according to Latif et al. (GenBank accession number L15409) in which the first nucleotide of the coding sequence is 214.

RESULTS

DNA samples from affected members of 93 consecutive VHL families were analyzed at the Genetic Diagnosis Laboratory of the University of Pennsylvania (Philadelphia, PA). Germline mutations were detected in affected members of 93/93 (100%) VHL families. Of the 78 distinct mutations detected, 8 (10%) were deletions of the entire VHL gene detected by quantitative Southern blotting, 23 (30%) were partial deletions detected by Southern blotting, and the remainder (n = 47; 60%) were point mutations detected by sequencing the VHL gene. A typical quantitative Southern blot from patients with complete or partial deletions of the VHL gene is presented in Figure 1. Point mutations, included 13 novel germline VHL mutations, are presented in Tables 1–3.

Deletions of the entire VHL gene detected by quantitative Southern blotting were verified by FISH studies of B-cells from affected individuals. Deletion of the entire VHL gene detected by Southern blotting was confirmed by FISH in eight unrelated, affected individuals (Pack et al., submitted). In these individuals, a single fluorescent signal was seen in B-cells hybridized with probes for the VHL gene.

We and other scientists have described genotype-phenotype correlations in VHL (Crossey et al., 1994; Chen et al., 1995; Zbar, 1995; Maher et al., 1996). Germline mutations predicted to inactivate the VHL protein were associated with renal carcinoma and CNS hemangioblastomas without phenochromocytoma (VHL type 1); germline mutations predicted to produce full-length VHL proteins were associated with pheochromocytoma in addition to the other manifestations of VHL (VHL type 2).

We identified germline VHL mutations in 67 VHL type 1 families. These mutations included 18 missense mutations, 11 nonsense mutations, 8 microdeletions, 2 insertions, 2 splice site mutations, 18 rearrangements, and 8 deletions of the entire VHL gene. Mutations in codon 167, the VHL hot spot, occurred in 2/67 VHL type 1 families. Eleven new germline VHL mutations were identified in the VHL type 1 families (Tables 1–3).

We identified germline mutations in 26 VHL type 2 families. These mutations included 18 missense mutations, 2 nonsense mutations, 1 microdeletion mutation, and 4 rearrangements. Germline mutations in codon 167 were identified in 12 VHL type 2 families. There were two new VHL mutations detected in VHL type 2 families. The frequency of missense mutations in VHL type 2 families (69%) was significantly different from the frequency of missense mutations in VHL type 1 families (27%) (<I>P</I> = .003). The frequency of codon 167 mutations in VHL type 2 families (46%) was significantly different from the frequency of codon 167 mutations in VHL type 1 families (3%) (<I>P</I> = .000002).

We have previously described large families with a characteristic VHL phenotype: pheochromocytoma without renal carcinoma, and classified these families as VHL type 2A (Chen et al., 1995; Zbar et al., 1996). In this study, we identified mutations Leu128Phe, Ser111Cys, and Arg161Gln in VHL families with four members affected with pheochromocytoma without renal carcinoma. Because the number of affected family members was small (n = 4), it was not possible to assign these mutations to a VHL type 2A phenotype.

DISCUSSION

In this article, we used methods that detect different types of mutations to improve the frequency of detection of germline mutations in the VHL families. These methods included quantitative Southern blotting to detect deletions of the entire VHL gene, Southern blotting to detect partial VHL gene deletions, fluorescence in situ hybridization to confirm deletions of the entire VHL gene, and complete sequencing of the VHL gene in patients in whom mutations were not detected by Southern blotting. With these methods we were able to

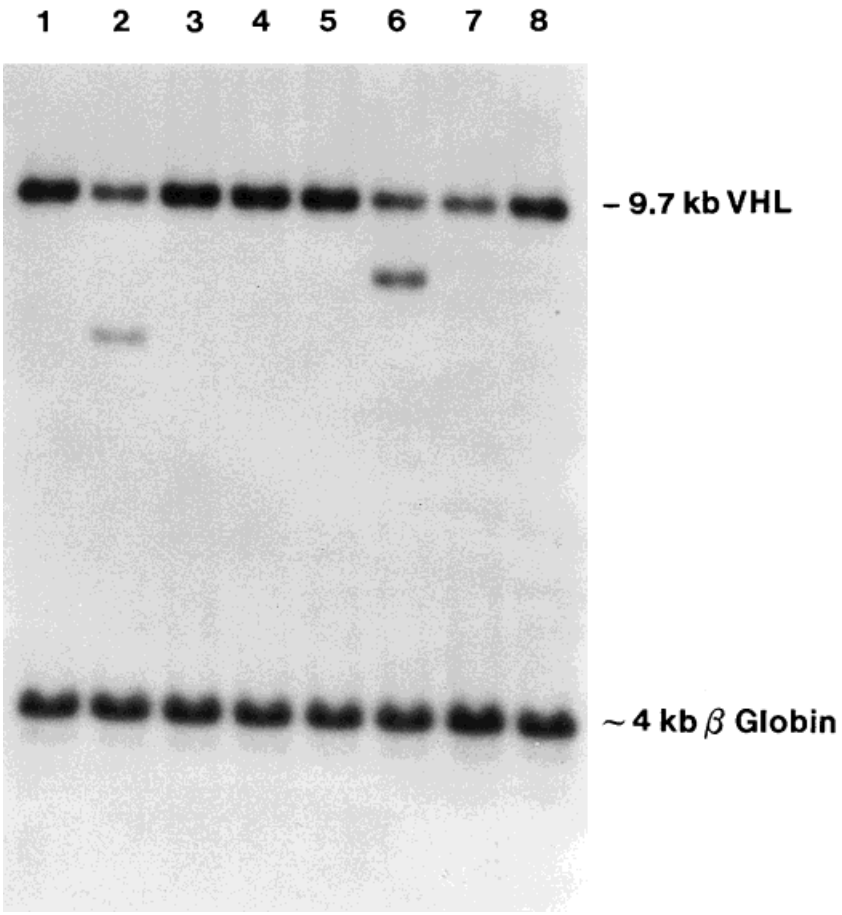

FIGURE 1. **Genomic DNA (5 μg) was digested with Eco RI and Ase I and separated by electrophoresis in 1 X TAE, and transferred to a nitrocellulose filter by capillary blotting. The filter was hybridized to random primer labeled probes specific for the VHL gene (gp 7) and the β globin gene (as an internal standard for DNA loading). With equal DNA loading, samples from control individuals (lanes 1,3,4,5, and 8) exhibit a single intense 9.7 kb band. Samples with a rearrangement in the VHL gene (lanes 2 and 6) exhibit a less intense 9.7 kb VHL band and an abnormally migrating band. Samples with a deletion of the VHL gene (lane 7) have a 9.7 kb band similar in intensity to that observed in rearranged samples.**

detect germline VHL mutations in 100% (93/93) of VHL families. This mutation detection percentage represents an improvement over previous mutation detection rates (Crossey et al., 1994; Chen et al., 1995; Maher et al., 1996; Glavac et al., 1996).

Several factors may explain the high VHL mutation detection rate achieved in our laboratory. First, the diagnosis of VHL in all families was confirmed by clinical examination at the National Cancer Institute (Bethesda, MD). No patients in whom the diagnosis of VHL was uncertain were included in this study. Inclusion of families in which the diagnosis of VHL was incorrect would

TABLE 1. **Types of Mutations Found in VHL Families Without (Type 1) and With (Type 2) Pheochromocytoma***

VHL type	No. of families	Missense	Codon 167	Nonsense	Microdeletion (1–18 bp)	Insertion (1–8 bp)	Splice site	Rearrangement	Deletion
1	67	18	2	11	8	2	2	18	8
2	26	18	12	2	1	0	0	5	0
Total	93	36	14	13	9	2	2	23	8

***The frequency of missense mutations in VHL type 1 was significantly different from the frequency of missense mutations in VHL type 2 ($P = .003$). The frequency of codon 167 mutations in VHL type 1 was significantly from the frequency of codon 167 mutations in VHL type 2 ($P = .000002$).**

TABLE 2. Summary of VHL Germline Mutations in Families Without Pheochromocytoma

Lineage	Mutation	Consequence	Type	
175*	393 delG	393 delG	Frameshift	
80	407 C→A	Ser65stop	Nonsense	
53	407 C→G	Ser65trp	Missense	
179	421 G→T	Glu70stop	Nonsense	
20*	430 C→T	Gln73stop	Nonsense	
183	439–441 del TTC	439-441delPhe	Microdeletion	
136	439–441 del TTC	439-441delPhe	Microdeletion	
132	440 T→C	Phe76Ser	Missense	New
43	454 C→T;775C→G	Pro81Ser	Missense	
128	470 C→G	Pro86Arg	Missense	
6*	475 T→A	Trp88Arg	Missense	
11	488-90 del 18bp	48-90 del18dp	Microdeletion	
48	499 C→T	Gln96Stop	Nonsense	
103	514-515 CT→GG	Leu101Gly	Missense	New
208	526 A→C	Thr Pro	Missense	
76	533 G→C	Arg107Pro	Missense	New
18*	545 G→A	Ser111Asn	Missense	
81	550 C→T	Arg113stop	Nonsense	
83	564 G→T	Trp117Cys	Missense	
146*	564 G→T	Trp117Cys	Missense	
13	565–566 insA	565insA	Frameshift	New
86	605 A→C	Asn131The	Missense	New
64	608 del A	608delA	Frameshift	New
119	643 del G	643delG	Frameshift	New
16	688 del A	688delA	Frameshift	New
22	694 C→T	Arg161stop	Nonsense	
184	694 C→T	Arg 161Stop	Nonsense	
93	694 C→T	Arg 161 Stop	Nonsense	
69	698 G→T	Cys162Phe	Missense	
141	703 C→T	Gln164Stop	Nonsense	
152	712 C→T	Arg167Gly	Missense	
97	712 C→T	Arg167Gly	Missense	
75*	715 ins 8 bp	715ins8bp	frameshift	
7	736 T→G	Tyr175Asp	Missense	New
25	753–756 del CGTC	753-756delCGTC	Frameshift	New
14	764 T→C	Leu184Pro	Missense	
50	768 C→G	Tyr185stop	Nonsense	
78	769 G→T	Glu176Stop	Nonsense	
161*	769 G→A	Glu186Lys	Missense	
21	insertGGT IVS			New
207	G→C +5 exon 1 spl			
130	Rearrange			
30*	Rearrange			
32*	Rearrange			
41	Rearrange			
45	Rearrange			
134	Rearrange			
210	Rearrange			
79*	Rearrange			
129	Rearrange			
108	Rearrange			
159*	Rearrange			
70	Rearrange			
24	Rearrange			
149*	Rearrange			
55*	Rearrange			
107	Rearrange			
147	Rearrange			
51*	Rearrange			
28	Deletion			
45	Deletion			
157	Deletion			
87	Deletion			
23	Deletion			
59*	Deletion			
38	Deletion			
5	Deletion			

* = Mutation (family) previously reported in Chen et al., 1995 or Zbar et al., 1996.

TABLE 3. Germline Mutations in VHL Families with Pheochromocytoma

Lineage	Mutation	Consequence	Type	
174	543–544 CA→TT	Ser111Cys	Missense	
12	553 G→C	Gly114Arg	Missense	
29*	594→595 GC→TT	Leu128Phe	Missense	
65	643 G→T	Gly144Stop	Nonsense	
92	694 C→T	Arg161Stop	Nonsense	
3	695 G→A	Arg161Gln	Missense	
150*	699 C→G	Cys162Trp	Missense	
202	712 C→T	Arg167Trp	Missense	
47*	712 C→T	Arg167Trp	Missense	
102	712 C→T	Arg167Trp	Missense	
106	712 C→T	Arg167Trp	Missense	
109*	712 C→T	Arg167Trp	Missense	
153	712 C→T	Arg167Trp	Missense	
100*	713 G→A	Arg167Gln	Missense	
42	713 G→A	Arg167Gln	Missense	
44	713 G→A	Arg167Gln	Missense	
88	713 G→A	Arg167Gln	Missense	
105*	713 G→A	Arg167Gln	Missense	
135	713 G→A	Arg167Gln	Missense	
115*	739delA	739 delA	Nonsense	
73	776 T→C	Leu188Pro	Missense	New
15	Rearrangement			
52	Rearrangement			
77*	Rearrangement			
186*	Rearrangement			
122*	Rearrangement			

*Mutation (family) previously reported in Chen et al., 1995 or Zbar et al., 1996.

lower the mutation detection rate. Second, the use of quantitative Southern blotting facilitated the detection of deletions of the entire VHL gene. Previously, deletions of the VHL gene region was detected by pulsed field gel electrophoresis (PFGE) (Richards et al., 1993; Yao et al. 1993) Third, the Eco RI/Ase I digest for Southern blotting provided better transfer of fragments and better resolution of partial gene deletion fragments than that achieved by digestion with Eco RI alone. Two patients in this study had rearrangements detectable by Eco RI/Ase digestion that were not detected after Eco RI digestion. Fourth, in research laboratories samples may not have been tested with methods that detect the different types of mutations that cause VHL. For example, some laboratories may report the results of mutation analyses based on single stranded DNA conformation analysis plus Southern blotting to detect VHL gene rearrangements without looking for complete gene deletions. Fifth, testing of individuals who are germline mosaic for VHL may fail to detect germline mutations. In two mosaic VHL families, it was necessary to test more than one affected family members to detect the germline mutation.

Of the 92 families shown to have germline VHL mutations, we detected partial VHL gene deletions in 23 (25%) families and complete VHL gene deletions in 8 (9%) families. Previous studies that looked for germline deletions by PFGE found a frequency of VHL gene deletions of 3% (Richards et al., 1993; Yao et al., 1993). While the frequency of germline deletion of the entire VHL gene is higher than predicted by the PFGE studies (3% vs. 9%), it is probably lower then determined in this series of patients. In a separate group of VHL patients, deletions of the gene were identified in 3 of 50 (6%) of probands (Stolle, unpublished observations).

Of the 13 new germline VHL mutations we detected 11 were associated with VHL type 1, and 2 were associated with VHL type 2. This observation suggests that the number of mutations that produce VHL associated with pheochromocytoma are more limited than the number of VHL mutations that produce VHL without pheochromocytoma.

Previous genotype-phenotype correlations observed in VHL were confirmed in this article. Mutations at codon 167 were found to be responsible for 46% of VHL type 2. Three mutations (Leu128Phe, Ser111Cys, and Arg161Gln) were identified that produced a clinical picture of pheochromocytoma without renal carcinoma. Continued observation of families with these mutations will be required to determine whether the observations in a limited number of affected individuals can be confirmed and extended.

The detection of mutations in 100% of VHL families has a number of implications. First, the

mutation data supports the concept that VHL is a genetically homogeneous disease. Second, when VHL mutation testing is performed using the approach described in this article, an individual not found to have a mutation is unlikely to be a VHL gene carrier. Third, disease producing mutations are unlikely to occur in the VHL 3′ UTR, 5′ UTR, or intervening sequences. If disease-producing mutations occurred in these regions of the VHL gene (regions not tested in this article), the mutation detection frequency would be less than 100%.

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